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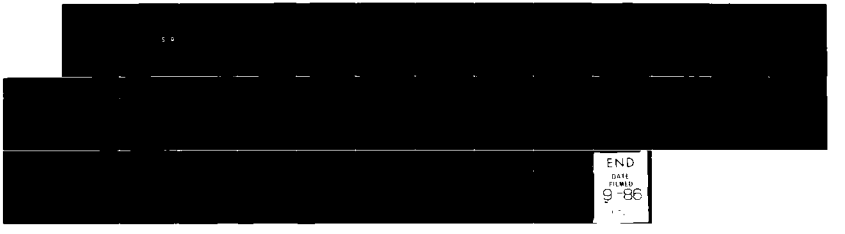

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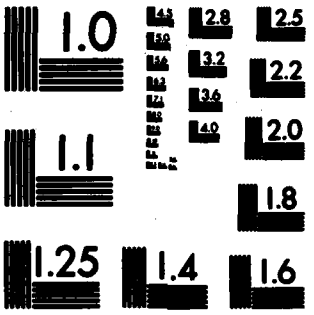

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# ENZYME MINI-TEST FOR FIELD IDENTIFICATION OF LEISHMANIA ISOLATES FROM U. S. MILITARY PERSONNEL

Annual Report

RICHARD D. KREUTZER

15 AUGUST 1984

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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It is possible to identify Leishmania isolates by cellulose acetate electrophoresis (CAE) of up to 29 enzyme activities. Certain of these enzymes are polymorphic within a subspecies and therefore of limited value for identification; others are monomorphic and have taxonomic significance. Once large numbers of isolates from various geographical areas have been characterized and monomorphic enzymes identified, a simple, rapid, accurate field type identification test can be devised.

To date approximately 200 Leishmania isolates have been characterized for up to 29 enzymes by CAE. About 150 of these isolates were primary isolates from the WRAIR cryobank and the remainder were from individual WRAIR investigators or contractees. Among the primary isolates were those designated by WHO as reference strains. Enzyme profiles based on reference strain isolates have been established by L. braziliensis panamensis (LBP), L. b. braziliensis (LBB), L. b. guyanensis (LBG) L. mexicana mexicana (LMM), L. m. aristedesi (LMAR), L. pifanoi (LPIF), L. m. enriettii (LME), L. garnhami (LGAR), L. donovani (LD), L. chagasi (LC), L. major (LMJ), L. tropica (LT), L. aethiopica (LAE), and L. hertigi hertigi. Initial characterization of L. mexicana amazonensis (LMA) and L. m. venezuelensis (LMV) has been completed but more isolates are necessary for definitive characterization. It has been noted that the numbers of Leishmania subspecies identified from a given area is related to the numbers of isolates examined; i.e. as more isolates from a particular geographic locale were studied the number of subspecies identified increased. A group of isolates from human hosts from Central America, some with simple cutaneous and others with diffuse cutaneous leishmaniasis (DCL), were over 75% identical, and therefore samples from one Leishmania subspecies. These data suggest that DCL is probably a result of host response to the parasite rather than due to differences in parasite strains. It is possible that these isolates are LMV. Identification of isolates should preceed studies on Leishmania; furthermore, confirmatory CAE identification should be made as such studies progress. Such identifications insure the reliability of the data obtained. Preliminary data indicate that two strains of visceral isolates, one derived from the other and one susceptable and the other resistant to $Sb^{+5}$ treatment can be separated by CAE. It is possible that specific enzyme polymorphism might be related to drug susceptibility. The test leading to accurate and rapid identification of Leishmania isolates requires analysis of enzymes which produce distinctly migrating bands for each subspecies, are monomorphic and are simple to run. The enzymes which appear to meet these requirements are GOT, GPI, $GSR_1$, $GSR_2$, ICD, MDH, MPI and 6PGDH. Simplified CAE conditions for these enzymes have been determined. A preliminary draft for the test has been proposed which includes the enzymes GPI, MPI, 6PGDH and $GSR_2$. The data generated in this study were combined in a dendrogram based on CAE similarities and differences within and between Leishmania species complexes. A new enzyme, $GSR_2$, and conditions for its CAE were reported. Technicians in the Leishmania section at WRAIR are already using the information provided in this report.

# ENZYME MINI-TEST FOR FIELD IDENTIFICATION OF LEISHMANIA ISOLATES FROM U. S. MILITARY PERSONNEL

Annual Report

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15 AUGUST 1984

Supported by

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## Problem

*Leishmania* isolates from any source can be accurately and rapidly identified by cellulose acetate electrophoresis (CAE). Studies using 29 enzyme activities have shown that certain enzymes are polymorphic and of little value in identification, while others are monomorphic and have taxonomic significance. It is, therefore, necessary to characterize isolates from a series of geographic areas by examining a number of activities to find the few monomorphic enzymes that can be used in a simple, rapid, accurate field identification test.

## Background

Electrophoretic study of gene-enzyme systems represents a powerful tool in systematics, genetics, ecology, ethology and applied biology. Studies on the systematic value of electrophoretic data reveal high levels of genetic similarity between conspecific populations, with up to 85% identity of their loci, while the percent of genetic similarities among closely related species are usually much lower.[1-4]

Recently enzyme electrophoresis has been used to identify parasites of the genus *Leishmania*.[5-11] One study reported that 21% of the enzymes tested from particular subspecies were polymorphic.[11] This suggests that isolates which have profiles greater than 75% identical are the same subspecies, and isolates which are less than 75% identical are parasites from different subspecies. The enzyme profiles of some isolates in this study were identical, but in most cases, the profiles among isolates in a given subspecies were different. *Leishmania* can be grouped into five major complexes according to enzyme profiles, *braziliensis*, *mexicana*, *donovani*, *tropica* and *hertigi*. These designations for the most part are consistent with the taxonomic categories used by others.[12] Within these complexes the isolates can be grouped

into subspecies with varying levels of allozyme identity. It has been reported that the L. braziliensis complex is composed of two subspecies, L. b. panamensis and L. b. braziliensis (= L. b. guyanensis). The L. mexicana complex has three subspecies, L. m. mexicana, L. m. amazonenesis and L. m. "venezuelensis". Minimal numbers of L. tropica and L. donovani have been examined and subspecies remain to be delineated. These data are consistent with those obtained by the principal investigator in an earlier less comprehensive study. Results of this study demonstrate that electrophoresis can be used for rapid and accurate subspecific identification of Leishmania.[11]

The data which have been reported on enzyme profiles of leishmanial groups have been obtained from small numbers of isolates. Among known species there is a certain amount of naturally occurring enzyme polymorphism. Isolates which have been identified as belonging to the same group by classical methods and their identification confirmed by CAE do have slight genetic differences as noted from their individual enzyme profiles. This type of population polymorphism is to be expected and has been noted in all groups of organisms which have been studied by electrophoresis.[1-4]

A taxonomic enzyme profile must include as much information on polymorphism as can be obtained. The problems which can result from establishing taxonomic enzyme profiles from small numbers of isolates are noted in the following example. If L. donovani WR130 from Khartoum and L. donovani WR352 from India are compared, 68% of 25 enzymes would be identical by CAE, but when the WR352 profile is compared to ten other L. donovani isolates, the levels of identity are greater. Furthermore, if the enzyme analysis were confined to MDH, ICD, GOT, ALAT, AK1, EST, ACP1, MPI (Table 1) there would have been 0% identity and the two isolates would have been considered as two different species complexes. Therefore, taxonomic profiles must be

based on many enzyme systems and on data from a large number of isolates representing the entire geographical distribution of the group.

The identification of Leishmania based on 29 genetic loci produces more accurate results; however, it should be possible to make a rapid and accurate identification using only 2 or 3 enzymes once the geographic area of isolation is known. For example the WRAIR isolates WR209 (L. b. panamensis), WR 359 (L. b. braziliensis), WR225 (L. m. mexicana), WR381 (L. b. "venezuelensis"), and WR285 (L. chagasi) are all from Panama. These isolates can be separated as follows:

| | |
|---|---|
| GPI | 209 and 359 are identical. This pair differs from 225, 381 and 285 which differ from one another. |
| ALAT | 359 and 285 are identical. This pair differs from all others. |
| MPI | Each differs one from another. |

Isolates from the Ft. Sherman area in Panama would require the use of WR209, WR225 and WR285 as reference subspecies. Any of the three enzymes could be used to establish that an unknown is L. m. mexicana and not the other subspecies, while MPI could be used to establish that the unknown was either L. b. panamensis or L. b. braziliensis. From this example it is evident that baseline studies on isozyme polymorphism are required to select the few taxonomically relevant enzymes to be used in a simplified identifcation procedure.

## Approach

This study was designed to establish methods which the clinical laboratory technician can follow in the field to rapidly and accurately identify leishmanial isolates from U. S. military personnel. At present, emphasis is placed on identification of New World and Kenyan isolates in support of military operations in Central America and USAMRU-Kenya.

Isolates which are grown in any medium that can support sufficient growth of promastigotes are examined by CAE using up to 29 enzyme systems. At least 20 different isolates of each subspecies with adequate histories are studied to obtain information on natural polymorphism. Standard enzyme profiles have been established in a previous study,[11] and unknown isolates are compared to these standard profiles. The enzyme profiles of each subspecies are compiled to determine which enzymes have taxonomic value.

Although the procedure for CAE identification of leishmanial isolates is simple, it should be possible to further reduce the time and effort required to rapidly and accurately identify isolates. A simple kit designed for a particular geographic area will be assembled. This kit will contain instructions, buffers, stains, controls, and information on interpretation of results.

Summary of Previous Year Progress

Work has progressed toward establishing a test for the rapid and accurate identification of Leishmania isolated from U. S. military personnel. Isolates from U. S. soldiers infected in Panama have been characterized using 29 enzymes by cellulose acetate electrophoresis (CAE). There are at least six subspecies of Leishmania which infect humans in that country: L. braziliensis panamensis (21 isolates), L. b. braziliensis (2 isolates), L. mexicana mexicana (2 isolates), L. mexicana peruviana (1 isolate), L. mexicana subspecies (1 isolate), and L. donovani chagasi (3 isolates). In addition, other isolates from patients infected in Central and South America and Kenya were identified.

It was noted that L. m. peruviana has an extensive distribution which includes Venezuela, Dominican Republic, Peru, Panama, Belize and possibly

Costa Rica. Isolates of this subgroup can be separated from other subspecies by the use of only two enzymes, GPI and MPI.

Preliminary identification was made from promastigotes cultured on and "picked" from blood agar medium without liquid overlay. This culture technique eliminates the requirements for a centrifuge needed for washing of promastigotes cultured in liquid medium. Promastigotes cultured in this way can be identified by study of GPI and MPI which are easily detected, have minimal polymorphism, and produce distinctly migrating bands for most subspecies. Preliminary studies indicate that buffer and stains for these two enzymes can be prepackaged to eliminate the need for the pH meter and balance in the field.

The CAE procedures developed at Youngstown State have been taught to technicians in the Leishmaniasis section at WRAIR and are regularly used in the clinical diagnosis of Leishmania in U. S. soldiers.

## Progress

### Isolates identified

In the initial phase of the project emphasis was placed on isolates from a single geographical locale, Panama. As the project continued, Leishmania isolates from other New World and some Old World areas were studied (Table 2).

Included in Table 2 are a series of isolates, WR560-568 and 347, which are reference strains recommended by WHO for use in biochemical characterization of Leishmania.[13] The CAE enzyme profiles of these isolates were compared with those previously established for each subspecies. In most cases the profiles of these reference strains and the established profiles were very similar (minor differences attributed to natural polymorphism accounted for most of the differences). There was, however, a major change in the L. tropica

profile previously reported[11] was based on limited data from few isolates. Recently the designation of L. tropica major and minor has been changed to L. major (rural) and L. tropica (urban).[12] The reference isolates, WR567, L. major (LMJ), WR564, L. tropica (LT) and WR565, L. aethiopica (LAE), were used to establish the profiles of these Leishmania species. The former L. tropica profile was actually that of L. major. The profiles of these three are about 30% identical with each other. The identification of L. tropica complex isolates shown in Table 2 is based on their similarities with the WHO reference strains. Two isolates (possibly originally from a single host) of L. m. enriettii (LME) were studied, and the profile of this subspecies was added to those already available. This subspecies has some similarity with the L. mexicana complex of species. An isolate of L. m. garnhami (LGAR) was also studied and its enzyme profile established. The profiles of LGAR and L. m. mexicana (LMM) are very similar, but it is possible to separate the single isolate of LGAR from other L. mexicana complex subspecies. It is not possible at present to suggest that LGAR is either identical or not identical to LMM.

A series of L. chagasi (LC) isolates is being analyzed (WR484, 485, 513, 514, 515, 517, 518, 519, 520), and the group enzyme profile compared to the Old World visceral profile of WR560 and others. Among the twenty-one enzymes tested on these LC isolates, all have a more cathodal band for ICD and MDH than do the Old World isolates. Therefore, it appears that New World and Old World visceral isolates can be separated by these two enzymes. Additional population studies of Old World visceral isolates are needed, as well as confirmatory data from New World isolates. Study of 30 Old World visceral isolates is to begin in Fall, 1984.

In summary enzyme profiles have been established for L. braziliensis panamensis (LBP), L. b. braziliensis (LBB), L. b. guyanensis (LBG), LMM, L. m. amazonensis (LMA) (more data are needed for this subspecies), L. m. aristedesi (LMAR), L. pifanoi (LPIF), L. m. enriettii (LME), L. garnhami (LGAR), L. donovani (LD), LC, LMF, LT, LAE and L. hertigi hertigi (LHH).

Leishmania diversity

As noted in a previous report there appears to be a direct relationship between the number of isolates characterized from a particular geographical locale and the number of subspecies detected in that area. Among 52 isolates from Panama, 20 from Brazil and 14 from Belize there is a higher level of subspecies diversity than among isolates from other geographical regions which have been less vigorously studied (Table 3). These supporting data on the relationship between diversity and numbers of isolates examined emphasizes the need to examine large numbers of isolates from all geographical areas from which leishmaniasis has been reported to obtain an accurate picture of the degree of Leishmania diversity.

Distinct subspecies

CAE data on certain isolates, WR348, 548, 549, 336, were combined with data from fourteen other Leishmania isolates from human hosts (6 from Dominican Republic, 5 from Venezuela, 3 from Belize, 1 each from Peru, Panama, Costa Rica and Mexico).[14] These isolates were over 75% identical one with another, and therefore appeared to be members of the same Leishmania subspecies. About half of the isolates produced simple uncomplicated lesions in their hosts, while the other half produced diffuse cutaneous (DCL) manifestations. The data indicated that the same parasite subspecies produced different clinical symptoms in different human hosts, or as previously

suggested, some clinical manifestations can be attributed to host immunological factors rather than to differences in the parasite itself.[15-19] The enzyme profile of these isolates was distinct from any other subspecies profile (compared to available WHO reference isolates), but was about 30-40% similar to the LMM, LMA and LPIF profiles, 20% similar with LMAR profiles and about 10% similar to the LME profile. The 18 isolates produced distinct allomorphs by CAE for ALAT, EST, GPI, GOT, $GSR_1$, $GSR_2$, PEP, LP, MDH, MPI and $PGM_1$. This group also had other similarities with a subspecies of the L. mexicana complex, LMV;[20-21] therefore, it is possible that these widely distributed isolates are LMV. Efforts are being made to obtain samples of LMV for analysis.

Routine identification for WRAIR contractees

In addition to the WRAIR primary isolates, samples for CAE identification were received from and identified for individual WRAIR personnel and contractees. These are noted in Table 2 as from Berman, Hanson, Keithly and Anthony. Other isolates from NIH, Tesh and Bonventre were also identified. The Colombian isolates from Tesh are now in the WRAIR cryobank. In each case the isolate identification was based on comparative data from previously identified WHO reference strains. In certain cases the CAE identification was made prior to initiation of other studies on the isolate and later follow up confirming identification was made during the course of studies by the contractee. It is suggested that groups involved in Leishmania research either establish CAE identification in their own laboratories or that identification confirmation be made at this laboratory prior to study. In addition CAE identification should be made a standard procedure in each project. Preliminary and confirmatory CAE identification is considered necessary, because certain isolates received at this laboratory from various sources and

labeled as a particular species were identified by CAE as being other than the indicated species or as mixed cultures. Identification by CAE is too simple and accurate not to take advantage of the process.

Enzyme polymorphism related to treatment

Two old world visceral isolates WR378 and WR555 (identified in Table 2 as G378 and G555) were obtained from Dr. William Hanson, University of Georgia. WR378 is the parent strain and was susceptable to $Sb^{+5}$ treatment. WR555 was derived from WR378 after several passages through hamsters and is resistant to the Sb treatment. These two strains are currently being analyzed by CAE. Data from 21 enzymes have been obtained which reveal that each produces a differently migrating band for $GSR_1$ (WR378 more cathodal than WR555), MI (WR555 more cathodal), and ICD (WR555 more cathodal). These preliminary data; the primary analysis is not yet complete and a second analysis must be made of two new samples from Dr. Hanson. It is interesting to speculate that there might be a correlation between specific parasite enzyme polymorphism and drug resistance. If the differences already noted are confirmed, other isolates with identical polymorphism to either WR378 or WR555 should be tested for sensitivity to Sb. It must be emphasized that these are preliminary data, and that there is only a suggestion that certain enzyme polymorphism can be associated with drug resistance.

Mini Test

General

Isolates of a particular subspecies have a high level of enzyme similarity (over 75%), but usually no two isolates are 100% identical. The allozyme differences among isolates is a result of natural polymorphism.

This polymorphism can be of importance when studying biological parameters other than identification, as noted above for the visceral isolates, WR378 and 555. Enzyme polymorphism can affect electrophoretic identification of Leishmania isolates. If, for example, 6PGDH were the only enzyme used for an identification, LPB, LBB and LBG could be separated even though two differently migrated bands have been observed among isolates of each subspecies (non-overlapping polymorphism). "LMV", LMM, LMA which are also polymorphic for 6PGDH and LMAR and LPIF could not be separated because their polymorphism is overlapping. Then 6PGDH can identify L. braziliensis subspecies but not L. mexicana species. Enzymes chosen for a mini test identification should have either no or non-overlapping polymorphism, but ones which can separate Leishmania species and subspecies. Another consideration is the choice of enzymes which are very active (i.e. produce bands with small numbers of cells) and which are relatively simple to prepare. One monomorphic enzyme, simply prepared, with distinctly migrating bands for each Leishmania subspecies would be sufficient, unfortunately no single enzyme yet studied meets all of these requirements.

Population enzyme polymorphism is probably the most difficult parameter to determine. It requires study of many isolates (ideally 20) from the entire distributional range of the subspecies, but for many Leishmania subspecies only a few isolates from a restricted geographical area have been studied. In this study attempts are being made to reduce possible errors in biochemical identification by collecting data from many isolates with well documented (if possible already identified) histories from multiple geographical areas, Tables 2 and 4. The data from the isolates already run indicate that enzyme polymorphism is either minor or not

present for the enzymes GOT, GPI, $GSR_1$, $GSR_2$, ICD, MDH, MPI and 6PGDH; furthermore, these enzymes can be used to separate most New and Old World Leishmania. These enzymes produce good activity from small numbers of cells, and buffer/strain components have been changed so each system can be preweighed, sent through the mail and requires only the addition of distilled water prior to use (Table 5). Although there are eight enzymes noted in the table, all will not be necessary in the final mini-test for Leishmania identification.

Preliminary draft of mini test

1. Cells needed for identification.
   A. The minimum number of cells needed has not yet been determined; however, visible masses of cells grown on and picked off blood agar medium in petri dishes without a liquid overlay are sufficient to produce activity with GPI, $GSR_1$, $GSR_2$, MPI and 6PGDH.
2. Cell preparation for CAE.
   A. Separate cells from growth medium at 1,000 g for 10 min. and pour off growth medium.
   B. Add 1 ml normal saline to the pellet (visible mass of cells).
   C. Carefully resuspend cells.
   D. Separate cells and saline at 1,000 g for 10 min. and remove all saline using a pipette if necessary.
   E. Add an appropriate amount of buffer (14 parts distilled water:1 part 0.1 M Tris, 0.1 M Maleic acid, 0.01 M EDTA, 0.01 M $MgCl_2$; pH to 7.4) to the cells. About 1/3 to 1/2 the size of the pellet.
   F. Resuspend the cells in the buffer with a vortex.

G. Rapidly freeze and thaw three times.

H. Separate lysate from cell debris at 1,000 g for 10 min.

I. With a pipette remove the lysate from the cell debris and store at $-70^{\circ}$ until needed.

3. Buffer/stain preparation and conditions for CAE. See Table 5.

4. Tentative sequence leading to identification.

A. Run GPI.

1. Separate "LMV", LB complex, LD complex, LMM, LMAR, LAE.

2. Identical band (LBP, LBB, LBG, LMJ, LT, LME), (LMM, LMA, LGAR), (LD complex), (LMAR, LPIF).

B. Run MPI.

1. Separate LBB, LT, LD complex, "LMV", LMM, LAR, LPIF, LAE.

2. Not separated LBB-LBG, LMJ-LMG.

C. Run 6 PGDH.

1. Separate LBP, LBB, LBG.

D. Run $GSR_2$.

1. Separate LMJ, LME.

This proposed mini-test is preliminary, and has been included to demonstrate the progress that has been made toward this major objective in the first half of the project. One problem area is controls. Much effort has been placed on finding "dead" controls, that is, commercially available enzymes or stains which will migrate to a particular level under the conditions of the CAE. Currently the controls being used are the WHO reference stains (when a band produced by an unknown migrates identically with a band produced by the control isolate, both and considered identical for that enzyme.) "Dead" controls are preferred. The final draft of the mini-test

will be complete and will include specific control data, diagrams of the profiles indicating what to expect, how to interpret the enzyme data, lists of other characterized isolates which are identical to the controls and other information which will allow the clinical technician to follow a minutely detailed standard operating procedure to identify an unknown isolate or regularly check Leishmania cultures. A complete analysis of at least 25 systems should be made of any new isolate, because it is possible that enzyme polymorphism not observed in the preliminary study could correlate with other biological parameters.

In summary it appears that the subspecies of Leishmania for which enzyme profiles are available can be separated by study of four enzymes, GPI, MPI, 6PGDH and $GSR_2$. These are active enzymes for which CAE procedures have been simplified and among which only minor enzyme polymorphism has been observed. Commercially available controls are preferred to those now being used. Most of these preliminary data on the mini test have already been made available to and are being used by personnel in the Leishmania section at WRAIR for isolate identification. The data will also be reported at a future meeting.[23]

Biochemical similarities among Leishmania

Data from the isolates studied have been combined and correlated. The levels of similarity and difference among subspecies has been calculated in a manner similar to that used for genetic identity and distance analysis in diploids,[1-3] (Table 6). These data can be used to produce a dendrogram or grouping of Leishmania subspecies. This type of analysis is more meaningful if it includes data from about 20 enzymes for about 20 isolates of each subspecies. The dendrogram (Fig. 1) includes some subspecies similarities

for LMAR, LPIF, LH, LAE, LME and LGAR based on data from one or two isolates; therefore, the similarities of these subspecies must at present be considered preliminary. The relationships of LMM and LMA to themselves and among the others in the L. mexicana complex are questionable, and attempts are being made to obtain a series of LMA isolates from human hosts. At this point in the study development of a reliable profile for LMA is of paramount importance. The profile of Old World visceral isolates shortly will include data from about 30 more isolates, and the differences noted above between LD and LC can be sustained by these additional data. No CAE data are available on L. d. infantum or certain other Leishmania New or Old World types such as L. b. peruviana, L. gerbilli and others.

Computer analysis

The CAE data which have been generated by this study can be placed in a computer program so they will be readily available to personnel at WRAIR. The data in their present form have been compiled for interpretation in this laboratory, but they must be reorganized for computer storage. Work on this phase of the project has only recently been initiated; therefore, little progress can be reported.

New enzyme

A new enzyme, glutathione reductase two ($GSR_2$), has been adapted to Leishmania. The conditions for electrophoresis and components of the buffer/stain are reported in Table 5. $GSR_1$ and $GSR_2$ appear to be different enzymes, because $GSR_1$ requires NADPH and $GSR_2$ requires NADH. If these two reduced coenzymes are combined in the substrate/stain, Leishmania isolates produce two very differently migrating dark bands of activity (not close migration as might be expected from heterozygotes). If each substrate/

stain is prepared separately as noted in Table 5, the $GSR_1$ band for "LMV" is more anodal than is the band for LMM, but the $GSR_2$ band for "LMV" is more cathodal than is the band for LMM. $GSR_2$ separates the LB complex of species from all others, it separates "LMV", LMM-LMA, LMAR, LPIF and LME subspecies from one another and from all others (LMM-LMA and LGAR are identical), LD and LC bands are the same but different from all others and LMJ and LT differ from one another and all others. Data from this enzyme are being included in isolate enzyme profiles.

Publications and reports at meetings

Kreutzer, R. D., N. Sourtay and P. B. McGreevy. 1983. New World diffuse cutaneous leishmaniasis:possibly one enzyme type. Annual meeting of Am. Soc. Trop. Med. Hyg.

McGreevy, P. B., R. D. Kreutzer, E. D. Franke, H. A. Stimson, C. N. Oster and L. D. Hendricks. 1983. Taxonomy, clinical pathology and prognosis of leishmaniasis in U. S. soldiers infected in Panama. Annual meeting Am. Soc. Trop. Med. Hyg.

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TABLE 1. Enzymes tested in this study.

| Enzyme | Enzyme Abbreviation |
|---|---|
| Oxidoreductases | |
| Lactate dehydrogenases | LDH |
| Malate dehydrogenase | MDH |
| Malic enzyme | ME |
| Isocitrate dehydrogenase | ICD |
| Phosphogluconate dehydrogenase | 6PGDH |
| Glucose-6-phosphate dehydrogenase | G6PDH |
| Glyceraldehyde-phosphate dehydrogenase | $GAPDH_1$, $GAPDH_2$ |
| Gluthione reductase | $GSR_1$, $GSR_2$ |
| Transferases | |
| Glutamate-oxaloacetate transaminase | GOT & ASAT |
| Glutamate-pyruvate transaminase | A AT |
| Hexokinase | HK |
| 6-Phosphofructokinase | FK |
| Adenylate kinase | AK |
| Guanylate kinase | GUK |
| Phosphoglucomutase | PGM |
| Hydrolases | |
| Esterases | EST |
| Acid phosphatase | ACP |
| Peptidases | PEP |
| Peptidase D | PEPD |
| Lyases | |
| Aldolase | ALD |
| Fumerate hydratase | FUM |
| Isomerases | |
| Mannose phosphate isomerase | MPI |
| Glucose phosphate isomerase | GPI |

TABLE 2. *Leishmania* isolates characterized during this report period.

| WRAIR No. | Category[1] | Parasite Species[2] | Host Species | Source | Locality | Other Designation |
|---|---|---|---|---|---|---|
| 077 | NWC | LBP | Man | USAMRU-P | Canal Zone | |
| 491 | NWC | LBP | Man | WRAIR | Panama | Reinertsen |
| 492 | NWC | LBP | Man | WRAIR | Panama | Reinertsen |
| 493 | NWC | LBP | Man | WRAIR | Panama | Reinertsen |
| 526 | NWC | LBP | Man | WRAIR | Panama | Dominquez |
| 535 | NWC | LBP | Man | WRAIR | Panama | Tyus |
| 539 | NWC | LBP | Man | WRAIR | Panama | Carrillo |
| 556 | NWC | LBP | Man | WRAIR | Panama | Evans |
| 566 | NWC | LBP | Man | Evans | Panama | LS94, LV44 |
| 568 | NWC | LBG | Man | Evans | Brazil | M4147, LV476 |
| 049 | NWC | LBB | Man | USAMRU-P | Canal Zone | Sablan, WR242 |
| 540 | NWC | LBB | Man | Evans | Belize | BZ10 |
| 541 | NWC | LBB | Man | Evans | Belize | BZ14 |
| 542 | NWC | LBB | Man | Evans | Belize | BZ15 |
| 543 | NWC | LBB | Man | Evans | Belize | BZ17 |
| 545 | NWC | LBB | Man | EVans | Belize | BZ25 |
| 557 | NWC | LBB | Man | Evans | Belize | BZ16 |
| 562 | NWC | LBB | Man | Evans | Brazil | LV436, M2903 |
| 348 | NWC | "LMV" | Man | Neal | Costa Rica | LV191 |
| 548 | NWC | "LMV" | Man | Evans | Belize | BZ18 |
| 549 | NWC | "LMV" | Man | Evans | Belize | BZ26 |
| 347 | NWC | LMM | *Nyctomys* | Neal | Belize | WR450, WR458, M379, LUMP1641-1965, LV4, L11, GML92 |
| 364 | NWC | LMA/LMM | Man | Keithly | Brazil | UISS150492, WR384, WR421 |
| 563 | NWC | LMA/LMM | *Lutzomyia* | Evans | Brazil | PH8, LV10 |
| 561 | NW | LHH | *Coendou* | Evans | Panama | LV42 |
| 206 | NWC | Lsp | Man | WRAIR | Brazil | Collier |
| 544 | NWC | Lsp | Man | Evans | Belize | BZ21 |
| 553 | NWC | Lsp | Man | Keystone | Ecuador | Chest |
| 554 | NWC | Lsp | Man | Keystone | Ecuador | Face |
| 560 | OWV | LD | Man | Evans | Ethiopia | WR354, HV3, L82, LRC-L133, LV9 |
| 564 | OWC | LT | Man | Evans | USSR | LRC-L39, LV357 |
| 565 | OWC | LAE | Man | Evans | Ethiopia | WR298, L100, LRC-L147, LV24 |
| 547 | OWC | LMJ | Man | R. Beach | Kenya | LRC-L137 |
| 551 | OWC | LMJ | Rodent | R. Beach | Kenya | NLB095 |
| 552 | OWC | LMJ | Sandfly | R. Beach | Kenya | NLB144 |
| 558 | OWC | LMJ | Man | Hendricks | Kenya | Beach, NLB173 |
| 559 | OWC | LMJ | Man | Hendricks | Kenya | Beach, NLB175 |
| 567 | OWC | LMJ | Man | Evans | Israel | LRC-L137, JERICHO II, LV561 |
| 550 | OWC | UNK | Sandfly | R. Beach | Israel | NLB136A |

TABLE 2. Leishmania isolates characterized during this report period. (Cont.)

| WRAIR No. | Category[1] | Parasite Species[2] | Host Species | Source | Locality | Other Designation |
|---|---|---|---|---|---|---|
| 007 | NWC | LBB | Man | USAMRU-P | Panama | Flores |
| 134 | NWC | LBP or G | Man | USAMRU-P | Panama | Acosta |
| 336 | DCL | "LMV" | Man | NIH | Dom. Rep. | Cecilio |
| 354 | OWV | LD | Man | Evans | Ethiopia | |
| 355 | OWC | LMJ | Man | ? | Jericho | |
| 357 | NWC | LMA | Man | Schnur | Brazil | |
| 409 | NWC | LBG | Man | Keithly | Surinam | Davis |
| 463 | NWC | LBG | Man | Evans | Brazil | Desouza |
| 467 | NWC | Lsp | Man | Tulane | Colombia | Calcedo |
| 578 | NWC | LBP or G | Man | WRAIR | Panama | Placenta |
| 579 | OWC | LT | ? | Sacks/NIH | ? | Clone 121 |
| 580 | NWC | LBB | Man | Neva | ? | MUCO |
| 581 | OWC | LT | Man | NIH | ? | Ackerman |
| 582 | OWC | LT | ? | Sacks/NIH | ? | Clone 12 |
| 584 | OWC | LT | ? | Sacks/NIH | Afghan | Niazy |
| 366 | NWC | LBG | Man | Zeledon | Costa Rica | LBO12, HSJD1 |
| 367 | NWC | LBB | Man | Keithly | Brazil | LTBO14, WR386 |
| 369 | NWC | LMM | Man | Zeledon | Costa Rica | LBO11, HSJD11 |
| 467 | NWC | LBP | Man | Tulane | Colombia | LTB 111 |
| 472 | NWC | LBB | Man | Marsden | Brazil | Aurelino |
| 484 | NWV | LD or LC | Man | Reed | UNK | Hevania, BA7 |
| 485 | NWV | LD or LC | Man | Reed | UNK | Wilton, BA1 |
| 513 | NWV | LD or LC | Man | Reed | Brazil | Risia, BA3 |
| 514 | NWV | LD or LC | Man | Reed | Brazil | Marcos, BA11 |
| 515 | NWV | LD or LC | Man | Reed | Brazil | Elson, BA12 |
| 516 | OL | LME | Guinea Pig | Weinstein | UNK | |
| 517 | NWV | LD or LC | Man | Reed | Brazil | Ranilson, BA4 |
| 518 | NWV | LD or LC | Man | Reed | Brazil | Jose, BA7 |
| 519 | NWV | LD or LC | Man | Reed | Brazil | Dominqos, BA8 |
| 520 | NWV | LD or LC | Man | Reed | Brazil | Francisco, BA9 |
| 529 | OL | LME | Guinea Pig | Weinstein | UNK | |
| 585 | NWC | LMM | | WRAIR | Panama | WR227 |
| 586 | NWC | LMJ | | WRAIR | ? | WR367, WR386 |
| 587 | NWC | LMJ | ? | WRAIR | ? | WR508 |
| Foot | ? | LMM | Hamster | WRAIR | Berman | |
| Spleen | ? | LMM | Hamster | WRAIR | Berman | |
| 173 | ? | LMJ | ? | WRAIR | Berman | |
| 220 | ? | LMJ | ? | WRAIR | Berman | |
| LTB | ? | LMJ | ? | WRAIR | Berman | |
| 420 | ? | LMJ | ? | WRAIR | Berman | |
| 15A | ? | LMJ | ? | WRAIR | Berman | |
| G128A | ? | LD or LC | ? | Hanson | | |
| G128B | ? | LD or LC | ? | Hanson | | |
| G128C | ? | LD or LC | ? | Hanson | | |
| G128D | ? | LD or LC | ? | Hanson | | |
| G378 | OWV | LD | Man | Hanson | Khartoum | Parent strain |
| G555 | OWV | LD | Man | Hanson | Khartoum | Resistant |
| G539A | NWC | LBP | Man | WRAIR | Panama | Carrillo |
| G539B | NWC | LBP | Man | WRAIR | Panama | |
| CB5/84 | NWC | "LMV" | ? | Keithly | ? | |
| CD5/85 | NWC | LMM | ? | Keithly | ? | |
| $CD_2$5/85 | NWC | LMM | ? | Keithly | ? | |

TABLE 2. *Leishmania* isolates characterized during this report period. (Cont.)

| WRAIR No. | Category[1] | Parasite Species[2] | Host Species | Source | Locality | Other Designation |
|---|---|---|---|---|---|---|
| M1142 | ? | LBG | ? | Keithly | ? | |
| M2904 | ? | LBG | ? | Keithly | ? | |
| CA | ? | LBG | ? | Keithly | ? | |
| CB | ? | LBG | ? | Keithly | ? | |
| M1287 | ? | LBB | ? | Keithly | ? | |
| CP | ? | LBG | ? | Keithly | ? | Wild |
| CX | ? | LBG | ? | Keithly | ? | Clone 8-2 |
| CY | ? | LBG | ? | Keithly | ? | Clone 8-3 |
| CZ | ? | LBG | ? | Keithly | ? | Clone 7-D |
| CAPCP | ? | LBC | ? | Keithly | ? | |
| $CBS_1P$ | ? | "LMV" | ? | Keithly | ? | |
| $CCS_6P$ | ? | LMM | ? | Keithly | ? | |
| CDPCP | ? | LD | ? | Keithly | ? | |
| $CES_5P$ | ? | LBB | ? | Keithly | ? | |
| $CFS_6P$ | ? | LMM | ? | Keithly | ? | |
| $CGS_1P$ | ? | LBB | ? | Keithly | ? | |
| $B_1$ | OWV | LD | Man | Marr | Ethiopia | |
| $B_2$ | OWV | LD | Man | Farrell | Ethiopia | |
| $B_3$ | OWV | LD | Man | Blackwell | Ethiopia | |
| 233* | NWC | "LMV" | Proechimys | Lainson | Brazil | WR302, LUMP1718-1899, LV78, M1845, LB016C, M1287 |
| 321* | NWC | LM | Man | UNK | Brazil | 211.497, GOTAS |
| 363* | NWC | "LMV" | Man | Lair | Brazil | |
| 374* | NWC | Lsp | Man | Beach | Surinam | Pepper Trail |
| 405* | NWC | LBB | Man | Keithly | Brazil | Ferreira, LTB082 |
| 473* | NWC | LBB | Man | Marsden | Brazil | Julio |
| 489* | NWC | LGAR | Man | Peters | Venezuela | JAP78, LUMP1568 |
| 508* | NWC | LML | Man | Marsden | Brazil | LTB0012 |
| 509* | NWC | "LMV" | Man | Marsden | Brazil | Marino, B05-2 |
| 601* | UNK | LD or C | UNK | UNK | UNK | |
| 604* | NWC | LBB | Man | UNK | Brazil | LTB558 |
| 607* | NWC | LBB | Man | UNK | Barbosa | LTB12, Mucosa |
| 608* | NWC | LBB | Man | UNK | Brazil | LTB300 |
| 609* | NWC | LBB | Man | UNK | Brazil | LTB559, Corte de Pedro |
| 610* | NWC | LBB | Man | UNK | Manause | |
| 611* | NWC | ?? | *Lutzomyia* | UNK | Brazil | |
| Y-JS* | NWC | "LMV" | Man | Tesh | Colombia | Sanchez |
| Y-BP* | NWC | LBP | Man | Tesh | Colombia | Posado |
| Y-LOM* | NWC | LBP | Man | Tesh | Colombia | Maturana |
| Y-LA* | NWC | LBP | Man | Tesh | Colombia | Artamide |
| A001* | UNK | LH | UNK | Anthony | UNK | |
| A002* | UNK | LH | UNK | Anthony | UNK | |

TABLE 2. Leishmania isolates characterized during this report period. (Cont.)

| WRAIR No. | Category[1] | Parasite Species[2] | Host Species | Source | Locality | Other Designation |
|---|---|---|---|---|---|---|
| A003* | UNK | LMJ | UNK | Anthony | UNK | |
| A004* | UNK | LMJ | UNK | Anthony | UNK | |
| A005* | UNK | Lsp | UNK | Anthony | UNK | |
| A006* | UNK | LMJ | UNK | Anthony | UNK | |
| A007* | UNK | LH | UNK | Anthony | UNK | |
| A008* | UNK | LMM | UNK | Anthony | UNK | |
| A009* | UNK | Lsp | UNK | Anthony | UNK | |
| BE001* | UNK | LMJ | UNK | Berman | UNK | Ullman, from amastigotes |
| BE002* | UNK | LMJ | UNK | Berman | UNK | 508B |
| BE003* | UNK | LMJ | UNK | Berman | UNK | Old pros. 508A |
| K001* | UNK | LMG | UNK | Keithly | New World | CUMC1 7/84 |
| K002* | UNK | LBB, LD | UNK | Keithly | New World | CUMC2 7/84 |
| K003* | UNK | LBB, LD | UNK | Keithly | Old World | CUMC3 7/84 |
| K004* | UNK | LBB | UNK | Keithly | New World | X 9/84 |
| K005* | UNK | LBB | UNK | Keithly | New World | Y 7/84 |
| 346* | NWC | "LMV" | Orizomys capito | Neal | Brazil | LV81, M1824 |

[1] LBP - braziliensis panamensis
LBG - b. guyanensis
LBB - b. braziliensis
"LMV" - possibly mexicana venezuelensis
LMM - m. mexicana
LMA - m. amazonensis
LHH - hertigi hertigi
LD - donovani
LT - tropica
LAE - aethiopica
LMJ - major
LC - chagasi
LME - m. enriettii
LGAR - m. garnhami

[2] CAE identification

*CAE analysis incomplete, some fewer than 10 enzymes

TABLE 3. Geographical diversity of Leishmania subspecies associated with intensity of study.

| Locale | Species Diversity | Number of Isolates Characterized* |
|---|---|---|
| Panama | BP, BB, MM, C, MAR, HH, MV | 52 |
| Brazil | BP, BB, BG, MM-MA, C | 20 |
| Belize | BP, BB, MM, MV | 14 |
| Peru | BB, MV | 2 |
| Costa Rica | MV, BG, MM | 4 |
| Dominican Republic | MV | 2 |
| Venezuela | MV, MPIF, GAR | 4 |
| Surinam | BG | 1 |
| Colombia | BP, MV | 4 |
| Kenya | D, MJ | 8 |
| Ethiopia | D, AE | 3 |
| USSR | T | 1 |
| Israel | MJ | 1 |
| Afghanistan | T | 1 |

*Data on primary isolates from the WRAIR cryobank. Data from other sources not included.

TABLE 4. Numbers of isolates in each subspecies or species of Leishmania tested in this study. Note: 20 of each type should be required to produce a valid profile of the group. Isolates are from the WRAIR cryobank. Isolates without reasonable histories or those from WRAIR which are currently being characterized are not included.

| Species or Subspecies | Number of Isolates |
|---|---|
| LBP | 40 |
| LBB | 18 |
| "LMV" | 10 |
| LMM | 9 |
| LC | 9 |
| LD Old World | 8 |
| LMJ Old World | 9 |
| LT Old World | 5 |
| LBG | 4 |
| LME | 2 |
| LAE Old World | 1 |
| LMAR | 1 |
| LPIF | 1 |
| LGAR | 1 |
| LHH | 1 |
| LMA* | 3 |

*At present no satisfactory CAE data are available on the LMA profile.

TABLE 5. CONDITIONS FOR ELECTROPHORESIS AND COMPONENTS FOR DEVELOPING ZYMOGRAMS.

| Enzyme | Cell Buffer | Membrane** Buffer | Voltage | Run Time (Minutes) | Reaction Buffer | Substrate/Stain Components* |
|---|---|---|---|---|---|---|
| GOT | 2 | 1:14 | 180 | 15 | A | Substrate: 100 mg L-Aspartic acid; 75 mg ∝-Keto-glutaric acid, readjust to pH 8.0; add 10 mg Pyridoxal-5-phosphate; pour this mixture with agar*** by itself in plates. Stain: 75 mg Fast blue BB. |
| GPI | 2 | 1:14 | 180 | 15 | B | 20 mg Fructose-6-phosphate; 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 60 mg EDTA; 25 units (1 flake) Glucose-6-phosphate dehydrogenase (Bakers yeast). |
| $GSR_1$[a] | 3 | 1:9 | 180 | 12 | C | 30 mg Oxidized Glutathione; 5 mg NADPH; < 1 mg 2, 6-Dichlorophenol-indophenol; 15 mg MTT. |
| $GSR_2$[a] | 3 | 1:9 | 200 | 15 | C | 30 mg Oxidized Glutathione; 5 mg NADH; < 1 mg 2, 6-Dichlorophenol-indophenol; 15 mg MTT. |
| ICD[a] | 3 | 1:14 | 180 | 15 | D | 100 mg DL-Isocitric acid ($Na_3$), readjust to pH 8.0; add 15 mg MTT; 15 mg β-Nicotinamide Adenine Dinucleotide phosphate (NADP); 10 mg PMS. |
| MDH[b] | 4 | 1:9 | 200 | 15 | E | 15 mg oxalacetic acetic; 15 mg β-NADH ($Na_2$ salt). |
| ME | 2 | 1:14 | 180 | 15 | F | 270 mg DL-Malic acid; 604 mg Tris; 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 25 mg $MnCl_2$. |
| MPI | 5 | 1:5 | 160 | 15 | G | 15 mg Mannose-6-phosphate; 10 mg NADP ($Na_2$); 10 mg MTT; 5 mg PMS; 1 mg Glucose phosphate isomerase; 15 units (1 flake) Glucose-6-phosphate dehydrogenase. |
| 6-PGDH[a] | 3 | 1:14 | 180 | 15 | B | 15 mg 6-Phosphogluconic acid ($Na_3$ salt); 15 mg MTT; 15 mg β-TPN; 10 mg PMS; 60 mg EDTA ($Na_2$ salt). |

Cell buffers:

1. .1 M Tris/.1 M Maleic acid/ .01 M EDTA ($Na_2$),/.01 M $MgCl_2$; adjust to pH 7.4 with 40% NaOH.
2. .1 M Tris/.05 M Maleic Acid/.01 M EDTA/.11 M Sodium phosphate dibasic (16 g/1ℓ), pH 7.4.
3. .7 M Sodium phosphate monobasic/.13 M Sodium phosphate dibasic, pH 7.0.
4. .29 M Tris/.09 M Citric acid monohydrate, pH 7.0.
5. .05 M Tris/.05 M Sodium phosphate monobasic, pH 7.5.

Reaction buffers:

A. 0.1 M Tris, adjust to pH 8.0 with 50% HCl.
B. 0.06 M Tris (7.28 g/1ℓ)/0.04 M Sodium phosphate monobasic (4.72 g/1ℓ), pH 8.0.
C. 0.25 M Tris (30.24 g/1ℓ)/0.103 M Sodium phosphate monobasic (12.34 g/1ℓ), pH 8.4.
D. 0.1 M Tris (12.11 g/1ℓ)/0.069 M Sodium phosphate monobasic (8.21 g/1ℓ), pH 8.0.
E. 0.018 M Sodium phosphate monobasic/0.082 M Sodium phosphate dibasic, pH 7.4.
F. 0.06 M Tris (7.28 g/1ℓ)/0.057 M Sodium phosphate monobasic (6.88 g/1ℓ), pH 7.5.
G. 0.1 M Tris (12.11 g/1ℓ)/0.101 M Sodium phosphate monobasic (12.12 g/1ℓ), pH 7.5.

Number of applications of the aliquot to the cellulose acetate plate:

Dilutions are made using 1 part buffer (0.1 M Tris/0.1 M Maleic acid/QOl M EDTA/0.01 M $MgCl_2$, pH adjusted to 7.4) and 14 parts distilled water.
1:1 dilution then 1X: GPI, ME.
1X : GOT, $GSR_1$, MDH, 6-PGDH.
2X : ICD

*To make 50 ml of stain (about 6 samples). All chemicals from Sigma.

**The membrane buffers are dilutions of 1 part cell buffer: ____distilled water.

***The CA plates after electrophoresis are placed on substrate plates for ± 12 min. at 37°C; then blotted dry and placed on the stain plates on which the bands are monitored.

[a]These systems require cooling during electrophoresis.

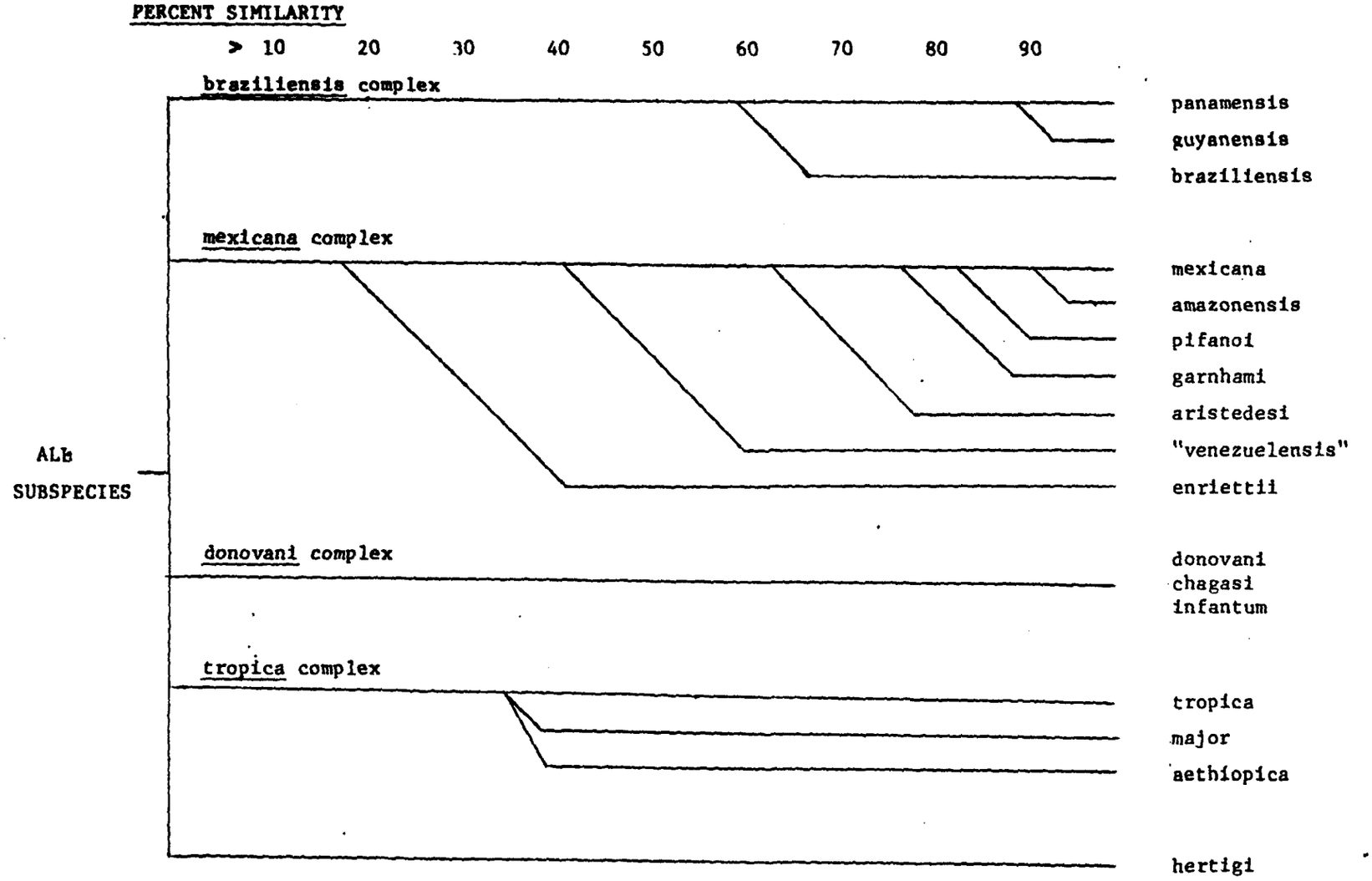
[b]These systems require viewing with Ultra-Violet light for the bands to be visible.

TABLE 6. Biochemical similarities among certain Leishmania subspecies and species. Some similarities are based on data from one isolate and at present must remain suspect. See Figure 1 for a graphic extension of these data.

| BP | BG | BB | | MJ | T | AE | |
|---|---|---|---|---|---|---|---|
| - | 89 | 59 | BP | - | 31 | 33 | MJ |
| | - | 58 | BG | | - | 33 | T |
| | | - | BB | | | - | AE |

| MM | MA | PIF | GAR | MAR | MV | ME | |
|---|---|---|---|---|---|---|---|
| - | 89 | 83 | 73 | 60 | 42 | 17 | MM |
| | - | 74 | 73 | 53 | 32 | 11 | MA |
| | | - | 60 | 53 | 35 | 11 | PIF |
| | | | - | 33 | 33 | 13 | GAR |
| | | | | - | 18 | 7 | MAR |
| | | | | | - | 11 | MV |
| | | | | | | - | ME |

Figure 1. Dendrogram grouping of subspecies of *Leishmania* based on their levels of enzyme profile similarities as noted in Table 6.



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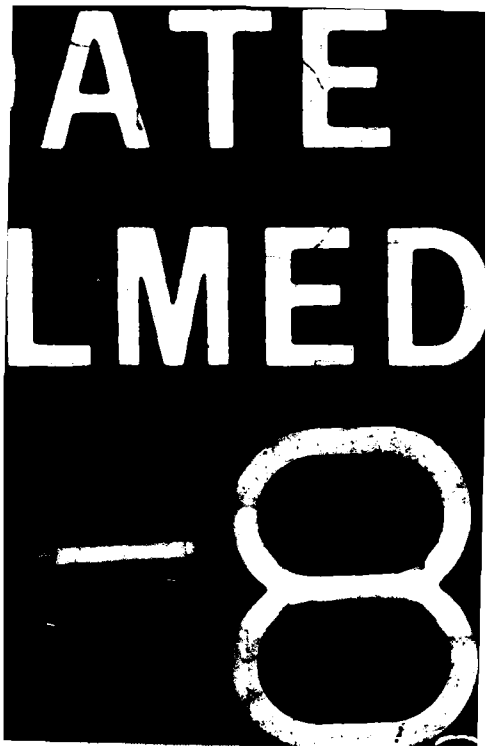
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